Plant Cell Reports (1987) 6:389–392

Plant Cell Reports

Indirect assessment of callus fresh weight by non-destructive methods

J. Mottley * and B. Keen **

ITAL Research Institute, P.O. Box 48, NL-6700 Wageningen, The Netherlands

Received April 21, 1987 / Revised version received July 22, 1987 – Communicated by W. Barz

ABSTRACT

The size of callus of Nicotiana plumbaginifolia was measured by determinations of fresh weight (FW), area (electronic planimeter and a point-counting method) and width (standard width and greatest width). All these methods, with the exception of the standard width measurements, were found to produce adequate substitutes for fresh weight.

Particular advantages apply to the use of the point-counting method, but the relationship between callus area and point interval was found to be critically important in determining the accuracy of measurements. The use of surface dimensions rather than FW permits continuous measurement of callus size without disturbance of the callus or its environment within the containers.

ABBREVIATION

FW: fresh weight

INTRODUCTION

Studies involving plant cell and tissue cultures often require growth measurements. For example, the optimization of media components, the effect of xenobiotics and other treatments, bioassays of plant growth hormones and comparisons of genotype performance all require some form of growth characterisation.

Most methods of assessing growth have been developed for use with suspension cultures (Street 1977; Gilissen et al. 1983; Grossman and Jung 1984; Davis et al. 1984), partly because low chemical gradients in the medium lead to high growth rates. The management of suspension cultures is, however, expensive in terms of labour, space and equipment. Several workers have, therefore, resorted to assessing growth of callus on solid medium, particularly when characterisation of large numbers of individual cultures is required.

Traditionally, fresh or dry weights are the main parameters measured (Horsch et al. 1980; Sloan and Camper 1981; Conner and Meredith 1984) but these involve either destructive sampling or repeated manipulation of the calli. This in turn leads to reduced or disturbed contact between the calli and the medium or intermittent alteration in the atmospheric composition of the containers. Some workers have tried to overcome these disadvantages by using surface area or volume of the callus as a basis for growth assessment. Fowler and Janick (1974) used a series of concentric circles of various sizes on transparent sheets to estimate callus diameters. They obtained a combined correlation coefficient of 0.95 between FW and either the diameter or the cube of the diameter. Nabors et al. (1982) obtained relative callus volumes by visually comparing callus size with clay spheres of various volumes but they did not provide data on the accuracy of the method nor the relationship between callus volume and weight. The accuracy of both these methods is severely limited by variations in callus shape which is always irregular and changes continously during growth.

There is clearly a need in the literature for a method for estimating callus size based on surface dimensions that is simple, rapid, essentially independent of shape, accurate and well-characterised with respect to the sizes and shapes of callus normally found in culture. One method we use to estimate area is to count the number of points on a grid placed at random over the base of the Petri dishes. The method is based on that used for estimating area sizes on maps (Frolov and Maling 1969; Barrett and Philbrook 1970; Bellhouse 1981), Visual Scene Analysis (Lloyd 1976) and ultrastructural analysis in cytology (Toth 1982). This point-counting method was compared with other non-destructive methods based on measurements of callus area, obtained with an electronic planimeter, and callus width.

MATERIALS AND METHODS

Cell Cultures : Callus cultures of Nicotiana plumbaginifolia (Viviana) were maintained on NH_4-S-RMOP medium (Marton et al. 1982) and routinely subcultured every month. At the start of each experiment 4 samples each of about 50 mg FW (mean ± S.D. = 50.9 ± 7.7 mg) were placed approximately equidistant on the surface of 36 plates of NH_4-S-RMOP agar medium and pressed slightly into the medium to obtain satisfactory

Present addresses:
* Department of Biology and Biochemistry, North East London Polytechnic, Romford Road, London E15 4LZ, UK
** Institute voor Toegepaste Informatica TNO, Postbus 100, NL-6700 AC, Wageningen, The Netherlands
Offprint requests to: J. Mottley

contact and to prevent the calli from becoming detached from the agar surface when taking size measurements. The plates were sealed with parafilm and incubated at a constant temperature of 28°C in the dark.

Size Measurements : Determinations of size were made on calli from 3 plates (12 calli) at each time interval and each callus was measured once only. The size parameters compared were callus area, obtained by the point-counting method of planimetry and an electronic planimeter, greatest width, standard width and FW.

Point counts were obtained by placing a transparent overlay at random on the base of the plate. The overlay had points traced on its surface, arranged in a square pattern at intervals of 5.0, 2.5 or 1.0 mm. A minimum of 6 points per callus area were used in each case. Only those points with their centres exactly on or inside the edge of each callus area were counted as 'in' and the areas of individual calli were calculated using the following formula:

$$A = N \times D^2$$

where A = calculated area, N = no. of 'in' points, and D = distance between the points. The practice of including all those points whose centres were exactly on the callus edge, rather than counting only half their number as 'in', should give a slight overestimate of the area but greatly increases the speed of area estimation.

Greatest width measurements were obtained by measuring with a ruler the distance between the two furthest points on each callus surface at any given time. Standard width measurements were made by measuring along a line drawn at random through each callus surface on the base of each Petri dish at the beginning of the experiment.

For electronic planimeter readings, the callus perimeters were first carefully traced onto a plastic transparency. Planimeter area readings were obtained by tracing along the perimeters of each callus trace using a MOP 30 (Kontron Messgerate GMBH, Munchen, FDR) with a point resolution of 0.1mm. Areas were automatically calculated and displayed.

After measuring surface dimensions, the FW of each callus was rapidly obtained using a top-pan electronic balance after removal of any adhering agar medium.

RESULTS AND DISCUSSION

Replacement of FW by Width and Electronic Planimeter Area Measurements

There were highly-significant correlations between ln FW and the ln of each of the three substitute measurements (electronic planimeter areas and the two widths) (Table 1) with approximately constant variation around the regression lines. The total error around the regression line is composed of the sum of the calibration and biological variation. Since these two sources of error are independent, the variance of the total error is the sum of the biological and calibration errors. The standard deviations of the biological variation were approximately constant if measurements were expressed on a ln scale (Fig.1). Extra variation around the mean curve implies that more observations are needed for establishing the growth curve with the same accuracy as with direct measurement of FW. The relative increase in variance due to calibration alone can be expressed as the relative increase in number of observations necessary to obtain the same accuracy as with direct measurement of FW (Keen 1986).

From Table 1 it can be seen that the calibration error caused by the accurate measurements of the substitutes is rather high. However, compared with the biological variation of approximately 47% found with our results (Keen 1986), a coefficient of variation of 13% and even 24% is acceptable in many situations. The extra number of observations required to achieve the same accuracy as FW measurements (8% for the electronic planimeter area and 25% for the greatest width) is worthwhile in practice. The standard width seems to be inadequate.

Influence of the Ratio of the Callus Size to Point Interval on the Accuracy of the Point-Counting Method

The standard error of a measurement of ln area obtained with the point-counting method (σ) will depend on the grid size in relation to the callus area in addition to the biological and calibration variances. Simple determination of σ requires a single and easily observed feature that is relevant for deciding which grid size to use for a given callus. Statistical analysis (Keen 1986) revealed that the relationship between the mean number of points per callus area (K) and σ is linear on the ln-ln scale. By plotting curves of σ with 95% confidence intervals against ln K it is possible to obtain a conservative estimate (upper curve), an optimistic estimate (lower curve) or a mean estimate (middle curve) of the minimum K value that produces a specified σ. A selection of values for the conservative estimate of σ at certain minimum K values is included in Table 2. The minimum value of K obtained can then be translated into a choice of grid size based on a preliminary rough estimate of the callus area.

The type of relationship between σ and K is the same as that derived by Frolov and Maling (1969) for regular shapes (circles, rectangles, etc.). The standard error for the point-counting method derived with regular shapes is, however, lower than the one derived from our experimental data for all sizes of calli and significantly lower at the 5% level for large calli.

Influence of the Point-Counting Error on the Calibration Relationship Between Planimeter Area and FW

The calibration error for an inaccurate measurement is the sum of the measurement error and the calibration error for the accurate measurement. Because both errors are independent the variance of the combined error is the sum of the variances of the separate errors. The consequences of calibration using the point-counting method have been summarised in Table 2. By comparison of Tables 1 and 2 it can be concluded for example that calibration using the point-counting method choosing the grid size such that K=9 is approximately as accurate as calibration using the greatest width. Both methods result in approximately 24% increase in number of observations needed for establishing the growth curve with the same accuracy as FW measurements. If K>9 then the accuracy of the point-counting method exceeds that using greatest width.

In conclusion, the four methods of measuring surface dimensions which have been compared in this report vary in their accuracy as substitutes for FW

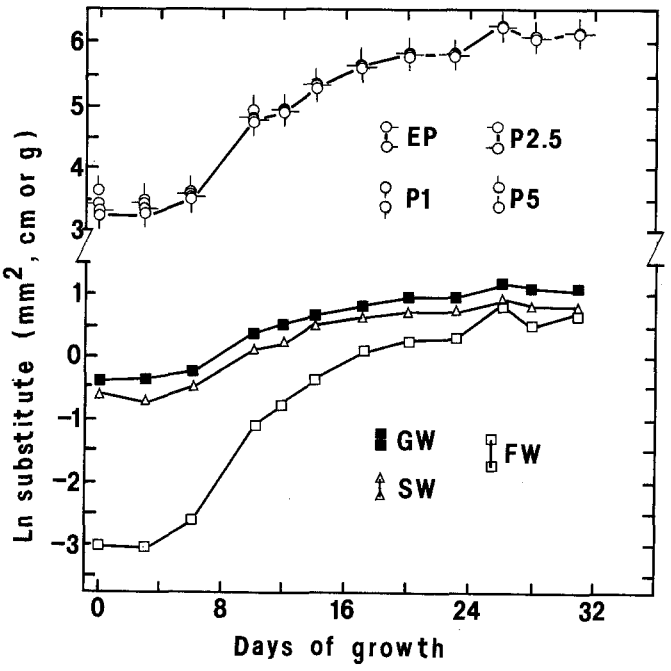

Figure 1. Growth curves of N. plumbaginifolia callus as assessed using FW and various substitutes. The abbreviations are as follows: EP, electronic planimeter area: P1, P2.5 and P5, point-counting areas using point distances of 1.0, 2.5 and 5.0 mm, respectively: GW, greatest width: SW, standard width: FW, fresh weight. The height of the vertical bars with symbols represents the standard deviation for each substitute measurement.

in callus growth studies. However, very high accuracy is not always the sole consideration, particularly when routine measurement of the size of large numbers of individual calli is required. Other considerations include expense of equipment, speed of measurement and disturbance of the growing calli. The most accurate substitute for FW found with our results is the electronimeter planimeter area but electronic planimeters are expensive, time-consuming to use and require special appliances for handling Petri dishes. Greatest width measurements, although acceptably accurate, can also be time-consuming, especially when the callus is relatively regular in shape. Standard diameters were found to be unsuitable in our study, possibly as a result of some degree of asymmetric growth of the calli.

The point counting method specified in this report gives a good estimate of callus fresh weight if applied correctly. Once the user is experienced in this technique it can be more rapid than all the other methods examined in this report. The method can be modified according to priorities of accuracy or speed by varying the minimum number of points per callus area (K).

The point counting technique can be used in any situation where discrete calli may be viewed clearly through either the base or top of containers. In addition, if small point distances or special ocular grids are used, it can be adapted for obtaining complete growth curves of microscopic colonies produced from small cell aggregates or single cells. Weight determinations are not possible in this case and the very irregular and asymmetric changes that are often encountered during the first few cell divisions of single cells would lead to large inaccuracies in diameter measurements.

It should be noted that the calibration of these indirect methods should be checked for different cell species and particularly if more accurate parameters of cell growth, such as dry weight or cell number, are used.

Table 1: Estimates of parameters that are relevant to calibration using electronic planimeter area and widths.

Description of parameter	Planimeter Area	Greatest Width	Standard Width
Correlation coefficient between ln substitute and ln FW measurements[a]	0.995	0.986	0.943
Coefficient of variation due to calibration alone (%)	13	24	50
Relative increase in variance of ln FW due to calibration (%)	8	25	114

[a]All correlation coefficients were significant at the $p<0.001$ level.

Table 2: Estimates of parameters that are relevant to calibration using the point-counting method. For the relation between σ and K the more conservative upper 95% confidence limits have been used.

Description of parameter	σ	0	.05	.10	.15	.20	.30
	K	∞	42	15	9	6	4
Coefficient of variation due to calibration alone (%)		13	15	18	23	29	40
Relative increase in variance of ln FW due to calibration (%)		8	10	15	24	37	74

ACKNOWLEDGEMENTS

The authors are greatly indebted to Dr J Rostron of the North East London Polytechnic, London, England for producing the computer graphics. Dr A V Roberts (North East London Polytechnic), Prof. Dr B de Groot and Dr A de laat (ITAL) are acknowledged for very stimulating discussions.

REFERENCES

Barrett JP, Philbrook JS (1970) J Forestry 69: 149-151
Bellhouse DR (1981) Biometrics 37: 303-312
Conner AJ, Meredith CP (1984) Plant Cell Tissue Organ Culture 3: 59-68
Davies DG, Stolzenberg RL, Dusky JA (1984) Weed Sci 32: 235-242
Fowler CW, Janick J (1974) Hort Sci 9: 552
Frolov YS, Maling DH (1969) Cart J 6: 21-35
Gilissen LJW, Hannisch ten Cate CH, Keen B (1983) Plant Cell Reports 2: 232-235
Grossman K, Jung J (1984) Plant Cell Reports 3: 156-158
Horsch RB, King J, Jones GE (1980) Can J Bot 58: 2402-2406
Keen B (1986) Technical and Preliminary Research Report, Research Institute ITAL, Wageningen
Lloyd PR (1976) Cart J 13: 22-26
Márton L, Dung TM, Mendel RR, Maliga P (1982) Mol Gen Get 182: 301-304
Nabors MW, Kroskey CS, McHugh DM (1982) Z Pflanzenphysiol 105: 341-349
Sloan ME, Camper ND (1981) Pest. Biochem. Physiol. 15: 201-208
Street HE (1977) In: Street HE (Ed) Botanical Monographs Vol 11, Blackwell Scientific Publications, Oxford, pp 61-102
Toth R (1982) Amer. J. Bot. 69: 1694-1706.